STRAWBERRY SUPPLEMENTATION LESSENS VASCULAR INFLAMMATION AND DYSFUNCTION DISPLAYED BY *db/db* MICE

by

Chrissa Petersen

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STATEMENT OF THESIS APPROVAL

The thesis of **Chrissa Petersen**

has been approved by the following supervisory committee members:

Anandh Babu Pon Velayutham	, Chair	**06/07/2017**
		Date Approved
John David Symons	, Member	**06/08/2017**
		Date Approved
Kuberan Balagurunathan	, Member	**06/07/2017**
		Date Approved

and by **Scott Summers** , Chair of

the Department of **Nutrition and Integrative Physiology**

and by David B. Kieda, Dean of The Graduate School.

ABSTRACT

Cardiovascular disease is 2-4-fold more prevalent in patients with diabetes. In diabetes, vascular inflammation and then endothelial dysfunction leads to the development of atherosclerosis. Studies have shown that the consumption of strawberry improves cardiovascular risk, but effects of strawberry on diabetic vasculature are unknown. We sought to determine whether dietary strawberry supplementation attenuates vascular inflammation and dysfunction in diabetic mice. Seven-week-old male diabetic *db/db* and control *db/+* mice consumed standard or supplemented chow containing 2.35% freeze-dried strawberry for 10 weeks. The strawberry dose was equivalent to two human servings of strawberries (160g) per day. Measurements after 10 weeks of treatment included metabolic variables, lipid peroxidation, blood pressure, vessel function, vascular inflammation, and mRNA expression of inflammatory cytokines. Diabetic mice exhibited an increased body weight, food intake, blood glucose, serum cholesterol and triglycerides, and lipid peroxidation with an impaired peripheral glucose homeostasis. Strawberry supplementation does not improve these variables in diabetic mice. Blood pressure was higher, relaxation to acetylcholine in arteries was impaired, and vascular inflammation was enhanced in diabetic versus control mice. However, strawberry supplementation reduces blood pressure, improves vascular dysfunction, and suppresses vascular inflammation in diabetic mice. Consistent with these findings, relative to results obtained from control animals, elevations of MCP-1, IL8, and VCAM-1 expression were greater in carotid artery

endothelial cells from diabetic mice, but were suppressed in strawberry-treated diabetic mice. In conclusion, dietary supplementation of strawberry attenuates indices of diabetes-induced vascular dysfunction without altering metabolic variables. This study provides evidence for further considering strawberry as an adjunct therapy to improve vascular complications associated with diabetes.

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ACKNOWLEDGEMENTS

This thesis could not have been accomplished without the thoughtful and thorough work of Dr. Anandh Babu Pon Velayutham who directed and organized the work. I also thank my committee members whose feedback was vital: Dr. Kuberan Balagurunathan and Dr. J. David Symons.

CHAPTER 1

INTRODUCTION

Diabetes mellitus and its comorbidities such as obesity and cardiovascular disease are leading contributors to death in the United States. Those who have diabetes mellitus are twice as likely to die of cardiovascular disease than those who do not have diabetes (National Diabetes Report CDC, 2014). These diseases are largely diseases of lifestyle and the risk of acquiring and worsening them can be decreased by alterations in nutrition and activity. Epidemiological studies focused on risk factors for these diseases indicate that there are differences in the diets and lifestyles of those at higher and lower risk for disease acquisition as well as disease progression (Diabetes Prevention Program Research Group, 2002). Research indicates that the consumption of fruits and vegetables is one of the best ways to decrease the risk of diabetes and cardiovascular disease.

1.1. Literature Review

1.1.1. Endothelial dysfunction contributes to vascular complications in diabetes

Diabetes greatly increases the risk of cardiovascular diseases such as atherosclerosis (Chistiakov, Orekhov, & Bobryshev, 2015; Tabit, Chung, Hamburg, & Vita, 2010). Hyperglycemia-induced endothelial dysfunction is a key event in the

pathogenesis of atherosclerosis in diabetes (Tabit, et al., 2010). The entire vascular system is lined by a single layer of endothelial cells called endothelium, which is a physical barrier and regulates biomolecular exchange that occurs between cells within and outside of blood (Cutler, Petersen, & Velayutham, 2017). Health of the vascular system is dependent on the maintenance of proper function of the endothelial layer. Endothelial function is critical in overall vascular function and progression of endothelial dysfunction could lead to irreversible damage to the vascular system (Chistiakov, et al., 2015; Su, 2015). Indeed, endothelial dysfunction is an often-overlooked problem that is at the center of many of the health problems in the Western world. In a study, half of women with chest pain were found to have endothelial dysfunction as tested by Flow Mediated Dilation (FMD) – a test of vessel reactivity when the vessel is presented with a burst of flow - the most common way to test for endothelial dysfunction (Johnson, et al., 2015). Endothelial function is of particular interest for those suffering from diabetes. Endothelial dysfunction is known to be involved in the pathogenesis of macrovascular as well as microvascular diseases in diabetes (Triggle & Ding, 2010). Macrovascular diseases include cardiovascular disease, peripheral artery disease, as well as cerebrovascular disease. Microvascular diseases include nephropathy, neuropathy, and retinopathy.

1.1.2. Role of vascular inflammation in endothelial dysfunction

In diabetes, high glucose-induced adhesion of monocytes to vascular endothelial cells and the subsequent vascular inflammation plays a pivotal role in the development of endothelial dysfunction and atherosclerosis (Chistiakov, et al., 2015; Kolluru, Bir, & Kevil, 2012; Menzaghi, 2013; Paneni, 2013; Tabit, et al., 2010). In a vessel experiencing

hyperglycemia, reactive oxygen species (ROS) modulate two important pathways; the first is through the activation of NFκB and the second is through the impairment of the endothelial nitric oxide synthase (eNOS)/ nitic oxide (NO) signaling pathway (Kolluru, et al., 2012). These signaling events induce vascular inflammation by increasing inflammatory chemokines and adhesion molecules, such as: interleukin-8 (IL-8), monocyte chemotactic protein-1 (MCP-1), vascular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-Selectin (Paneni, 2013). Monocytes are a subset of leukocytes that make up around 5.3% of white blood cells. During homeostatic conditions, in large part, monocytes flow unimpeded through the bloodstream. When inflammation is present, monocytes within blood vessels are recruited to the area and are induced to associate with the endothelium. Once associated with the endothelium, the monocytes roll which is mediated by P-selectin and E-selectin. They then become attached to the endothelial cells. This process is mediated by VCAM-1 and ICAM-1. In inflammatory conditions, endothelial cells have weaker intercellular junctions and increased permeability leading to transmigration of monocytes to the subendothelial space that is mediated by MCP-1 (Steyers & Miller, 2014). Once the monocytes are in the subendothelial space, they take up oxidized low-density lipoprotein (LDL) particles and become foam cells. The foam cells contribute to the development of atherosclerosis. Endothelial dysfunction and atherosclerosis, therefore, can be retarded by the reduction of monocyte binding and their transendothelial migration. Berry anthocyanins could be one of the important dietary components that can ameliorate vascular complications in diabetes by reducing vascular inflammation.

1.1.3. Cardiovascular benefits of berry anthocyanins

Flavonoids are a diverse group of polyphenolic compounds found ubiquitously in plants. Epidemiological, clinical, and animal studies support the beneficial health effects of dietary flavonoids (Cutler, et al., 2017; Hooper, et al., 2008). There are six main classes of flavonoids: anthocyanidins (in berries and kidney beans), Flavan-3-ols (in tea and cocoa), flavones (in apples and celery), flavonols (in onions and romaine lettuce), flavanones (in oranges and grapefruit), and isoflavones (in soy and green beans) (Cutler, et al., 2017). Anthocyanins are a form of anthocyanidin which contains a sugar moiety. Anthocyanidins are colorless and as a fruit ripens, sugar is added to make anthocyanins which contribute a red or blue color to ripe fruits and to flowers. Anthocyanins are widely distributed in berries such as strawberries, blueberries, and raspberries and are also in other fruits and vegetables such as red radishes, grapes, and spinach (Cutler, et al., 2017). There are six major anthocyanidins: pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin. Sugars that conjugate with them include arabinose, galactose, glucose, and rhamnose. The variety of combinations will generate many types of anthocyanins (Basu & Lyons, 2010; Michalska & Łysiak, 2015). The amount and type of anthocyanins in food depend on the type of food as well as the conditions of the food's environment (Michalska & Łysiak, 2015). People consume around 12.5 mg of anthocyanins per day on average according to the National Health and Nutrition Education Survey (NHANES) (Wu, et al., 2006). The most common source of anthocyanins in the United States is from berry consumption. Berry consumption has positive cardiovascular effects (Basu, et al., 2010). The health benefits of anthocyanins are ascribed to their antioxidant, anti-inflammatory, antihypertensive, antiatherosclerotic, antimicrobial, anticancer, and neuroprotective

properties (Basu, et al., 2010; Cutler, et al., 2017). Strawberry and blueberries are the most commonly consumed berries in the US (Wu, et al., 2006).

1.1.4. Do strawberry anthocyanins improve vascular complications in diabetes?

Strawberry is an excellent source of health-promoting bioactive components such as anthocyanins and the anthocyanins most commonly found in strawberries are the glycosidic derivatives of pelargonidin and cyanidin. Pelargonidin makes up 90% of the anthocyanidin measured in strawberries with cyanidin making up almost all of the remaining 10% (Da Silva, Escribano-Bailón, Alonso, Rivas-Gonzalo, & Santos-Buelga, 2007). Strawberries tend to retain their bioactives during processing, including freeze drying and freezing (Azzini, et al., 2010; Truchado, et al., 2011). Epidemiological studies point to a correlation between consumption of strawberries and other berry fruits and their benefit to human health. The Nurses' Health Study looked at the diet and health outcomes for more than ninety-three thousand women. A significant negative correlation was found between regular (three times per week) strawberry and blueberry consumption and incident myocardial infarction (Cassidy, et al., 2013). In this study, regular consumption of blueberries and strawberries, two of the richest sources of anthocyanins in modern Western diets (Wu, et al., 2006), was associated with a 34% reduction in myocardial infarction risk compared to infrequent consumption of those fruits (Cassidy, et al., 2013). Of note, no significant association was found between the intake of other flavonoid subclasses and myocardial risk. Strawberry consumption has been correlated with positive vascular outcomes and has been studied for its role in inflammation and blood lipids. Human clinical

trials found that there was a significant decrease in total cholesterol, LDL cholesterol, and oxidized LDL upon consumption of strawberries and strawberry products (Alvarez-Suarez, et al., 2014; Basu, et al., 2014; Park, et al., 2016). One study found a difference in small particle LDL cholesterol, and lipid peroxidation upon supplementation with either the equivalent of 250 or 500 g of fresh strawberries in freeze-dried form (Basu, et al., 2014). However, the effects of strawberries on endothelial inflammation and dysfunction in general and in diabetes are unknown.

Anthocyanins are extensively metabolized in humans by the digestive enzymes and intestinal microbiota, suggesting the vascular effects could be mediated by their circulating metabolites (Kuntz, et al., 2016). Even though the parent anthocyanin compounds are found in low amounts in serum, metabolites of anthocyanins are likely involved in the positive effects of berry fruits consumption on cardiovascular disease (He & Giusti, 2010). Pelargonidin-3-O-glucuronide (P3G) is the major metabolite of the most abundant anthocyanin pelargonidin-3-O-glucoside. P3G reaches its highest concentration of 227 nM after consumption of 200 g strawberries by humans (Mullen, Edwards, Serafini, & Crozier, 2008). A carbon 13 clinical study found that some metabolites remain in human bodies for around 48 hours (Czank, et al., 2013). In addition, three phenolic metabolites such as coumaric acid (370 nM), 4 hydroxy benzoic acid (2.5 μM), and protocatecheuic acid (170 nM) appear in the plasma following the consumption of strawberries (Azzini, et al., 2010; Mullen, et al., 2008).

1.2. Purpose Statement

Diabetes has a major impact on cardiovascular diseases such as atherosclerosis and endothelial dysfunction and plays a role in the development of vascular complications. Hence, compounds that attenuate endothelial dysfunction can improve vascular complications in diabetes. Our study investigated the effect of supplementation of strawberry at a nutritional dose on vascular inflammation and dysfunction in diabetes. Diabetic *db/db* mice (an established model to study vascular complications in diabetes) and control *db/+* mice were fed with a standard chow or chow supplemented with 2.35% freeze-dried strawberry powder for 10 weeks. The following three hypotheses were tested.

1.2.1. Hypothesis 1

Dietary supplementation of strawberry reduces blood pressure and improves endothelial dysfunction in diabetic mice. To test this hypothesis, blood pressure and ex vivo vessel function were measured. Metabolic parameters were also assessed to determine whether the vascular effects of strawberry are mediated through improved metabolic parameters.

1.2.2. Hypothesis 2

Dietary supplementation of strawberry reduces vascular inflammation in diabetic mice and the vascular effect of strawberry is endothelial specific. To test this hypothesis, monocyte binding to the aortic vessel was determined. In addition, the mRNA expression of inflammatory chemokines and adhesion molecules in endothelial cells isolated from carotid artery of experimental mice was assessed.

CHAPTER 2

METHODS

2.1. Experimental Design

2.1.1. Experimental animals

Male diabetic *db/db* mice with a C57BLKS/J background (*db/db*; B6.Cg-m$^{+/+}$Lepr^{db}) as well as control *db/+* mice with the same background were ordered from the Jackson Laboratories, USA (Stock no. 000642). *db/db* mice are from a well-characterized strain of inbred mutant mice that have a novel splice site that leads to a truncated leptin receptor protein that lacks almost all of the usual intracellular domain. This is a widely-used type 2 diabetic animal model that spontaneously develops vascular complications (Babu, Si, Fu, Zhen, & Liu, 2012; Babu, Si, & Liu, 2012). The mice were held in the animal facility at the University of Utah and acclimated for a week before experiments were performed. Mice were exposed to experimental conditions beginning at 7 weeks old. All mice were held under humane conditions in the animal facility at the University of Utah. Animals were maintained under artificial light in a 12-hour light/ dark cycle, 23 ± 1° C, and 45 ± 5% humidity. Mice were housed in cages of 2-5 animals throughout the experiment. The Institutional Animal Care and Use Committee at the University of Utah reviewed the proposal for appropriate use of the animals and to approve the number of mice used.

2.1.2. Standard chow and strawberry supplemented chow

The customized pelleted diets were prepared as shown in Table 1 and were supplied by Dyets Inc. The standard chow was adjusted to compensate for the additional sugars provided by the freeze-dried strawberry. The diets were stored at -20 °C and thawed immediately before use. The amount of freeze-dried strawberry powder used in this study was calculated based on the Food and Drug Administration recommendation for the extrapolation of doses from animals to humans by normalization to body surface area (Nair & Jacob, 2016). The nutritional dose of freeze-dried strawberry powder was based on average human consumption. The amount of freeze-dried strawberry powder in diet was 2.35% (w/w), equivalent to two human servings of fresh strawberries (~160 g or 2 cups).

2.1.3. Segregation of experimental animals

After 1 week of environmental acclimation, diabetic mice (7 weeks old) were divided into two groups and received standard chow (n=15) or 2.35% freeze-dried strawberry supplemented chow (n=15) for 10 weeks. Control mice were divided into two groups and received standard chow (n=15) or 2.35% freeze-dried strawberry supplemented chow (n=10) for 10 weeks.

2.2. Measurement of metabolic parameters

The mice were weighed weekly as was their food. Blood glucose concentrations in tail vein blood samples were measured using a glucometer. The fasting blood glucose and non-fasting blood glucose were measured at week 10. Intraperitoneal glucose tolerance test (IPGTT) and intraperitoneal insulin tolerance test (IPITT) were conducted following 10

weeks of treatment. To analyze IPGTT, mice were fasted overnight and then injected with a single bolus of glucose (2 g/kg body weight) followed by the measurement of blood glucose concentrations at 0, 15, 30, 60, and 120 min after glucose administration. To perform IPITT, mice were fasted for 4 h and were injected with insulin (0.75 units/ kg body weight) and blood glucose concentration was measured at 0, 15, 30, 60, and 120 min after insulin injection. Serum cholesterol and triglycerides were measured using commercial assay kits according to the manufacturer's instructions (Abcam).

2.3. Measurement of lipid peroxidation

Serum lipid peroxidation was measured by measurement of hydroperoxides using a commercial assay kit according to manufacturer's instructions (Abcam).

2.4. Blood pressure measurement

Blood pressure was measured in conscious mice using a computerized, noninvasive blood pressure system by tail cuff method at week 10 (Kent Scientific Blood Pressure system) as described previously (Babu, Si, & Fu, 2012; Babu, Si, & Liu, 2012; Bharath, et al., 2015). The mice were acclimated to a holder while placed on a warming platform for at least 10 min to stabilize their temperature. An occlusion cuff and a pressure measurement cuff were then placed on the tail and blood pressure measurements began to be collected with the CODA software.

2.5. Assessment of vascular function

Mesenteric arteries were collected and the arterial reactivity was measured using a myograph as described previously (Bharath, et al., 2015). This experiment was carried out in Dr. J David Symons' laboratory at the University of Utah. The arteries were tested for stiffness with the Multi Wire Myograph System – 620M by Danish Myo Technology (DMT), Denmark. This test has been shown to test arterial stiffness with high reliability (Bharath, et al., 2015).

2.6. Assessment of vascular inflammation

The effects of dietary supplementation of strawberries on vascular inflammation in diabetic mice were assessed by measuring the binding of monocytes to the aortic vessel. The determination of monocyte adhesion to the aortic vessel was conducted using fluorescence-labeled WEHI-78/24 mouse monocytic cells (Babu, Si, Fu, et al., 2012; Babu, Si, & Liu, 2012). Aortas from the experimental mice were rapidly excised under general anesthesia. Fat and connective tissue was carefully removed, and the aortas were washed twice with ice-cold PBS. The aortas were placed in DMEM for 10 min at 37°C. The abdominal aorta segments, near the iliac, were used. The segments were then opened longitudinally to expose the endothelium and pinned onto 4% agar in 35-mm plates with 1 ml of EBM-medium containing 1% heat-inactivated FBS. WEHI monocytes were fluorescence labeled with calcein-AM (Invitrogen). The aortas were incubated for 30 min with fluorescence-labeled WEHI 78/24 mouse monocytes. After incubation, unbound monocytes were gently washed and the numbers of monocytes firmly bound to aorta were captured using confocal microscopy. Ten images were captured for each vessel. Ten

images of ten frames were captured for each vessel using Olympus confocal microscopy at 10X magnification. The number of monocyte bound to aortae were counted in a minimum of five fields per aorta and at least aorta from five mice per group. Data were represented as the mean ± SE.

2.7. Assessment of endothelial-specific effects of dietary strawberry using endothelial cells from carotid artery

To assess whether the effects of strawberry on vessels is endothelial specific, endothelial cells were further interrogated for markers of inflammation. Endothelial cells were isolated from carotid arteries by the method developed by Nam, et al. (2010). Briefly, carotid arteries were excised and perfused with QIAzol reagent (Qiagen). The vessel effluent containing the intimal fraction contains the endothelial cells and the remaining components of the vessel contain media and adventitia. The purity of endothelial cells in the vessel effluent was confirmed by assessing the expression of PECAM-1 which is specific to endothelial cells. Then the mRNA expression of inflammatory molecules such as MCP-1/JE and IL-8/KC and adhesion molecules VCAM-1, ICAM-1, and E-selectin were measured by qPCR. Briefly, total RNA was isolated from aortic vessels (Qiagen RNAeasy Plus mini kit), cDNA was synthesized (Qiagen RT-PCR kit), and the expression of these inflammatory molecules was measured by qPCR by using SYBR green (Qiagen, CA) as previously described (Muthusamy, et al., 2012).

2.8. Data analysis

Data are presented as mean ± SE, and $P < 0.05$ was considered different. Comparison of one time point among groups was made using one-way ANOVA with SPSS/10. Comparison of multiple time points among groups was made using a one-way or two-way repeated-measures ANOVA using Prism. Tukey post hoc tests were performed when significant main effects were obtained.

Table 1.

Strawberry-supplemented chow and standard chow

	Standard chow	Chow supplemented with freeze dried strawberry (2.35% in diet)
Ingredient	**g/kg**	**g/kg**
Casein, High Nitrogen	200	200
L-Cystine	3	3
Sucrose	89	85
Cornstarch	397.5	394
Dyetrose	132	132
Corn Oil	70	70
Cellulose	50	45
Mineral Mix (Dyet #210025)	35	35
Vitamin Mix (Dyet #310025)	10	10
Choline Bitartrate	2.5	2.5
Dextrose	5	0
Fructose	6	0
Freeze-Dried Strawberry	0	23.5
	1000	1000

CHAPTER 3

RESULTS

3.1. Strawberry supplementation does not change metabolic parameters in diabetic mice

Body weight, food intake, fasting blood glucose, non-fasting blood glucose, serum cholesterol, and serum triglycerides were greater in diabetic mice compared with control mice (Fig. 1A-D; Fig. 2A and B). However, strawberry supplementation did not change these metabolic variables nor the measured lipid levels in diabetic mice (Fig. 1A-D; Fig. 2A and B). The peripheral glucose homeostasis was assessed by glucose and insulin tolerance tests. Diabetic mice exhibited a severe impairment in peripheral glucose homeostasis (Fig. 3A and B). Strawberry supplementation failed to improve glucose homeostasis in diabetic mice (Fig. 3A and B).

3.2. Strawberry supplementation does not reduce lipid peroxidation in diabetic mice

Lipid hydroperoxidation as measured by the concentration of serum lipid hydroperoxides was significantly increased in diabetic mice compared to control mice (Fig. 4). Though strawberry supplementation modestly reduces lipid hydroperoxides in diabetic

mice, the decrease was not significant (p=0.058) (Fig. 4).

3.3. Strawberry supplementation reduces blood pressure in diabetic mice

Arterial pressure (systolic, diastolic, and mean) was significantly increased in diabetic mice compared to control mice (Fig. 5). However, strawberry supplementation reduces the severity of hypertension in diabetic mice (Fig. 5).

3.4. Strawberry supplementation improves endothelium-dependent vasorelaxation in diabetic mice

Vascular function was measured using a wire myograph. Relaxation to acetylcholine (10^{-8}–10^{-6}) in arteries precontracted with 10^{-6} phenylephrine was impaired in diabetic mice as compared to control mice. Strawberry supplementation ameliorates the endothelial specific dysfunction in diabetic mice as shown by increased vessel relaxation (Fig. 6A). This is in contrast to the endothelial independent vessel relaxation as measured by sodium nitroprusside, which was similar among groups (Fig. 6B).

3.5. Strawberry supplementation reduces vascular inflammation in diabetic mice

Monocyte binding to vessel was used to assess the vascular inflammation. There is an enhanced binding of WEHI 78/24 mouse monocyte to the aortic vessel isolated from diabetic mice as compared to control mice (Fig. 7). However, strawberry consumption reduces the binding of monocytes to the aortic vessel from diabetic mice (Fig. 7).

3.6. Strawberry supplementation reduces inflammatory chemokines and adhesion molecules in arterial endothelial cells isolated from diabetic mice

The endothelial-specific effects of strawberry bioactives were assessed by measuring inflammatory chemokines and adhesion molecules in the endothelial cells isolated from carotid arteries of experimental mice. The purity and the presence of endothelial cells in intimal fractions were confirmed by the presence of PECAM-1 (Fig. 8A). Endothelial cells isolated from the carotid arteries of diabetic mice exhibited an increase in mRNA expression of IL8/KC, MCP1/JE, ICAM1, and VCAM1 as compared to the carotid endothelial cells of control mice (Fig. 8B and 8C). However, the mRNA expression of IL8/KC, MCP1/JE, and VCAM1 was significantly reduced in strawberry-treated diabetic mice. This indicates the presence of an endothelial-specific anti-inflammatory effect of strawberry bioactives (Fig. 8B and 8C).

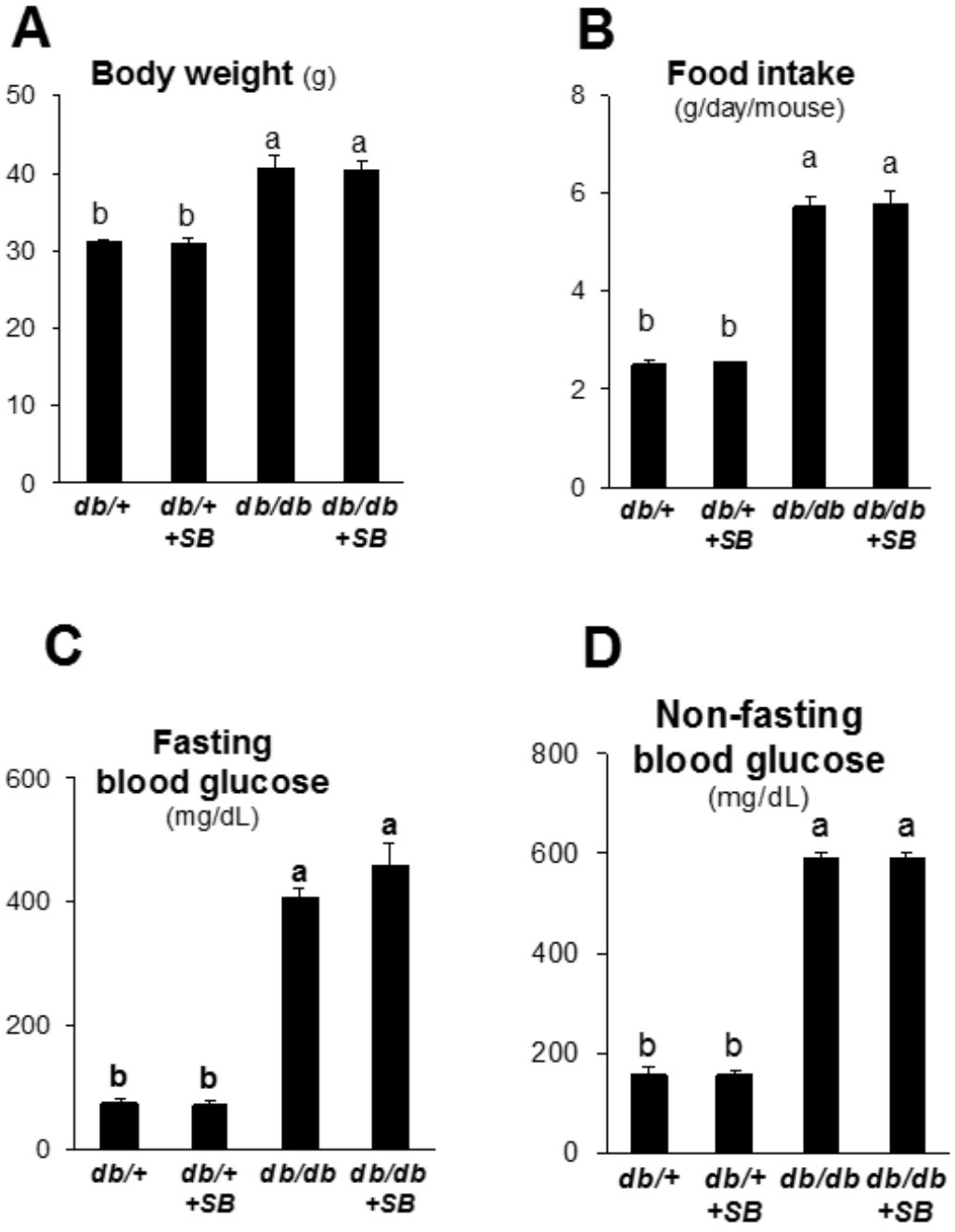

Figure 1. Metabolic Parameters. Strawberry supplementation does not change body weight *(n=10-15)* (A), food intake *(n=10-15)* (B), fasting blood glucose *(n=8-10)* (C), non-fasting blood glucose *(n=8-10)* (D) in diabetic mice. *db/+*: control mice; *db/+*+SB: control mice treated with strawberry; *db/db*: diabetic mice; *db/db*+SB: diabetic mice treated with strawberry. Values are mean ± SEM. Means without a common letter differ, $P < 0.05$.

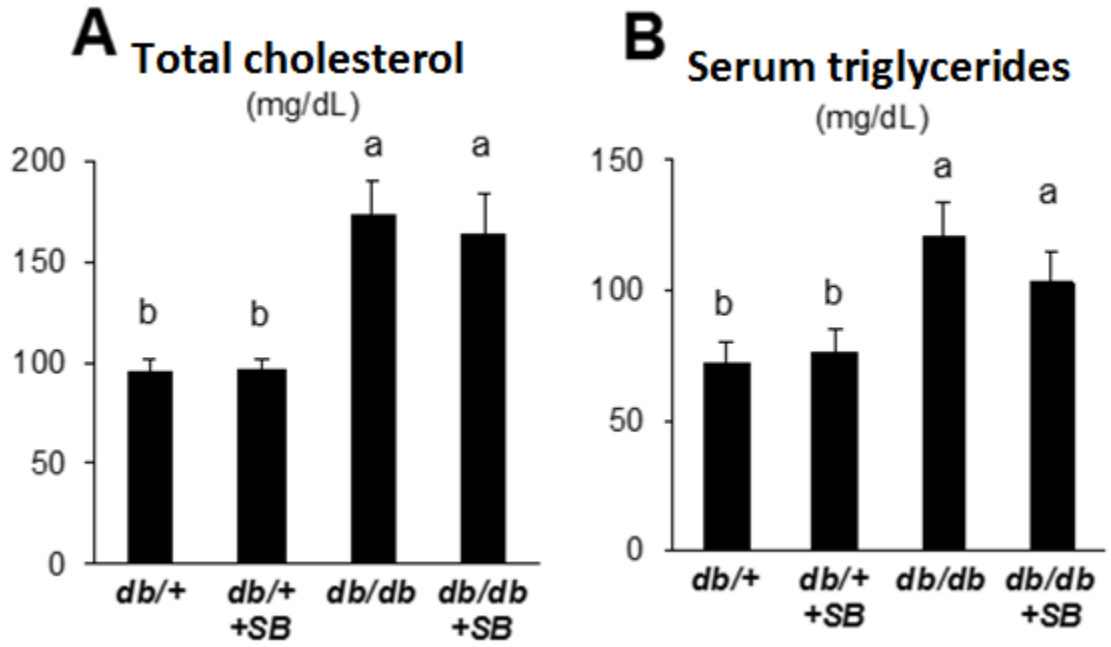

Figure 2. Lipids. Strawberry supplementation does not change serum total cholesterol *(n=7-8)* (A), and serum triglycerides *(n=7-8)* (B) in diabetic mice. *db/+*: control mice; *db/+*+SB: control mice treated with strawberry; *db/db*: diabetic mice; *db/db*+SB: diabetic mice treated with strawberry. Values are mean ± SEM. Means without a common letter differ, $P < 0.05$.

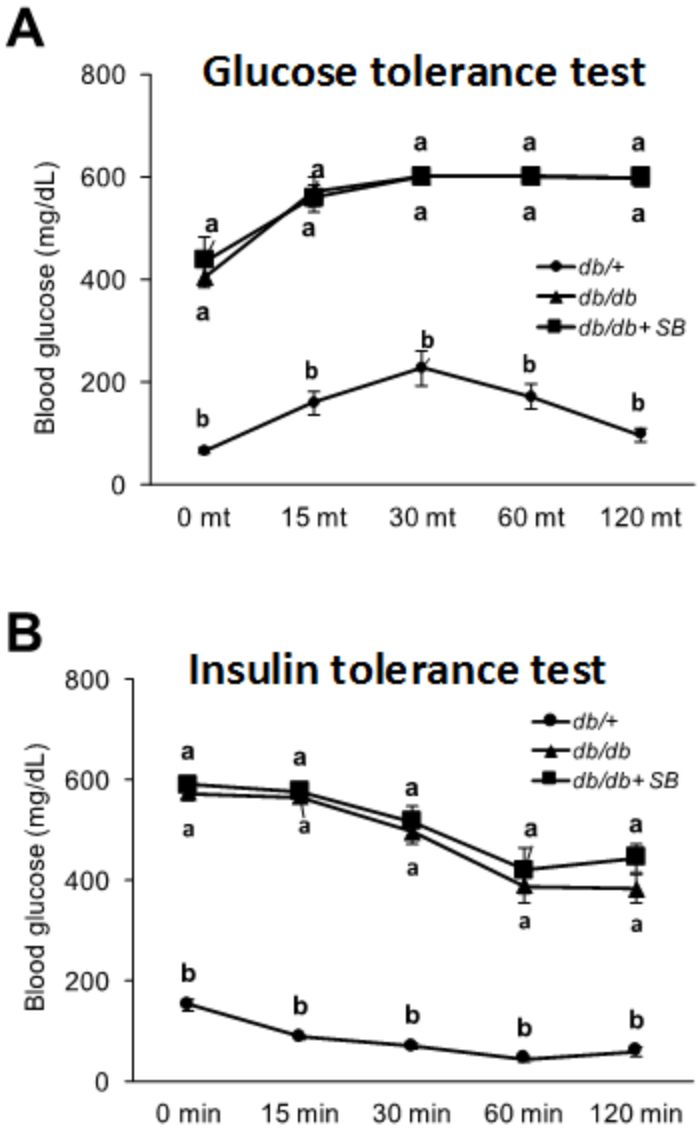

Figure 3. Glucose Tolerance Test and Insulin Tolerance Test. Strawberry supplementation does not improve glucose tolerance *(n=7-9)* (A), and insulin tolerance *(n=7-9)* (B), in diabetic mice. *db/+*: control mice; *db/+*+SB: control mice treated with strawberry; *db/db*: diabetic mice; *db/db*+SB: diabetic mice treated with strawberry. Values are mean ± SEM. Means without a common letter differ, $P < 0.05$.

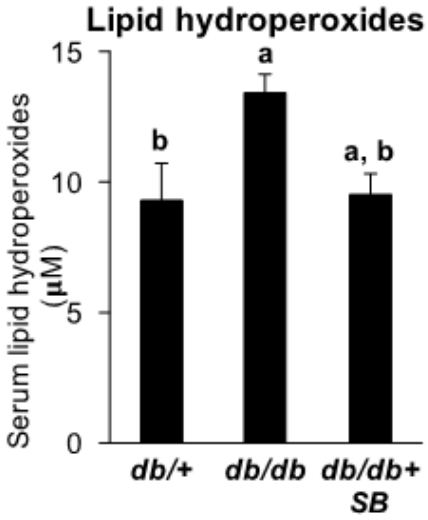

Figure 4. Lipid Peroxidation. Strawberry supplementation does not improve serum lipid hydroperoxides *(n=5-6)* in diabetic mice. *db/+*: control mice; *db/+*+SB: control mice treated with strawberry; *db/db*: diabetic mice; *db/db*+SB: diabetic mice treated with strawberry. Values are mean ± SEM. Means without a common letter differ, $P < 0.05$.

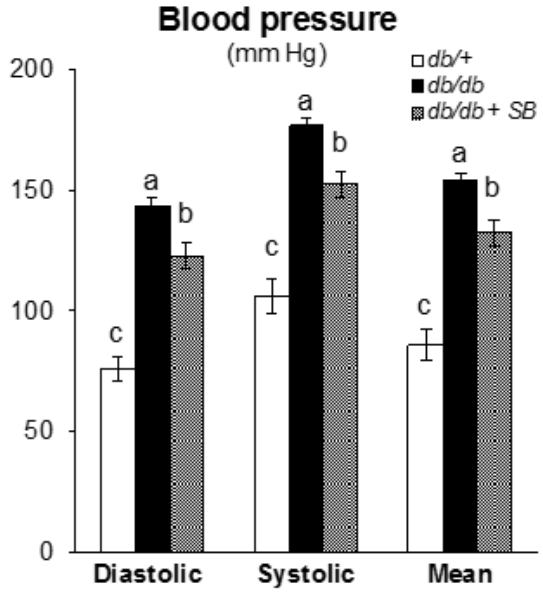

Figure 5. Blood Pressure. Strawberry supplementation reduces blood pressure *(n=7-9)* in diabetic mice. *db/+*: control mice; *db/db*: diabetic mice; *db/db*+SB: diabetic mice treated with strawberry. Values are mean ± SEM. Means without a common letter differ, $P < 0.05$.

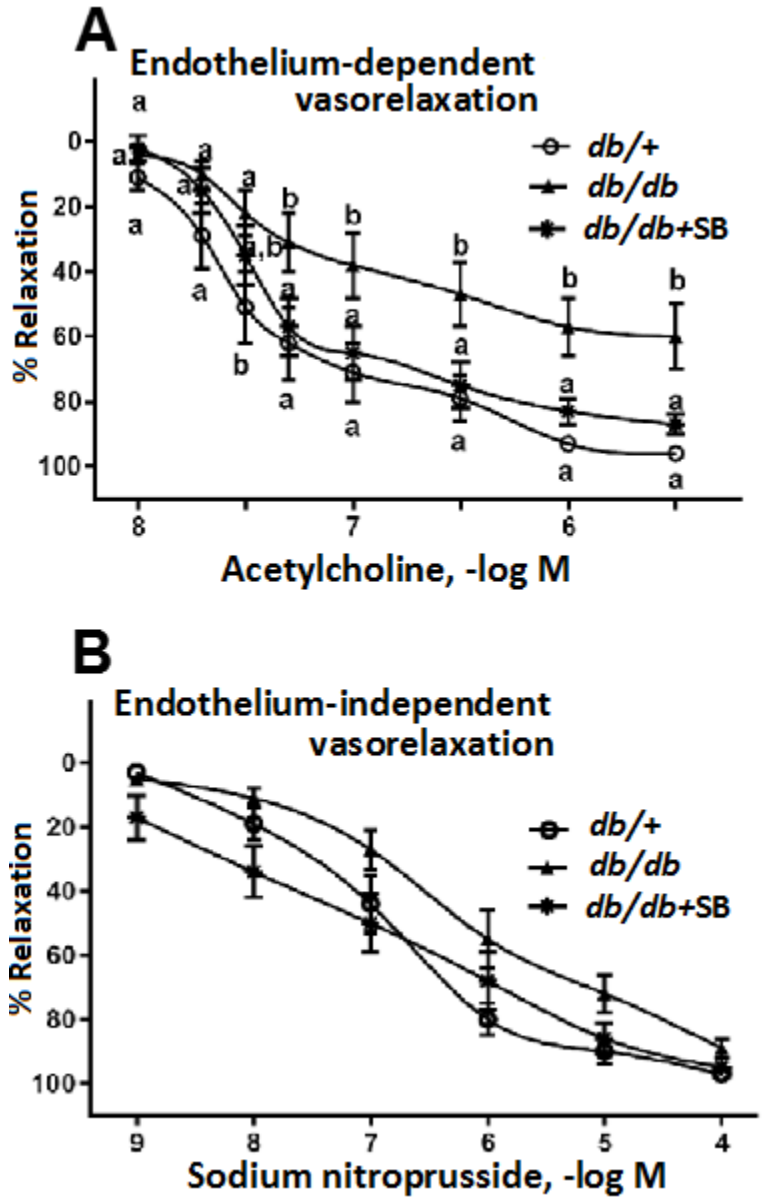

Figure 6. Vascular Function. Strawberry supplementation improves endothelium-dependent vasorelaxation *(n=6)* (A) without changing endothelium-dependent vasorelaxation *(n=6)* (B) in diabetic mice. *db/+*: control mice; *db/db*: diabetic mice; *db/db*+SB: diabetic mice treated with strawberry. Values are mean ± SEM. Means without a common letter differ, $P < 0.05$.

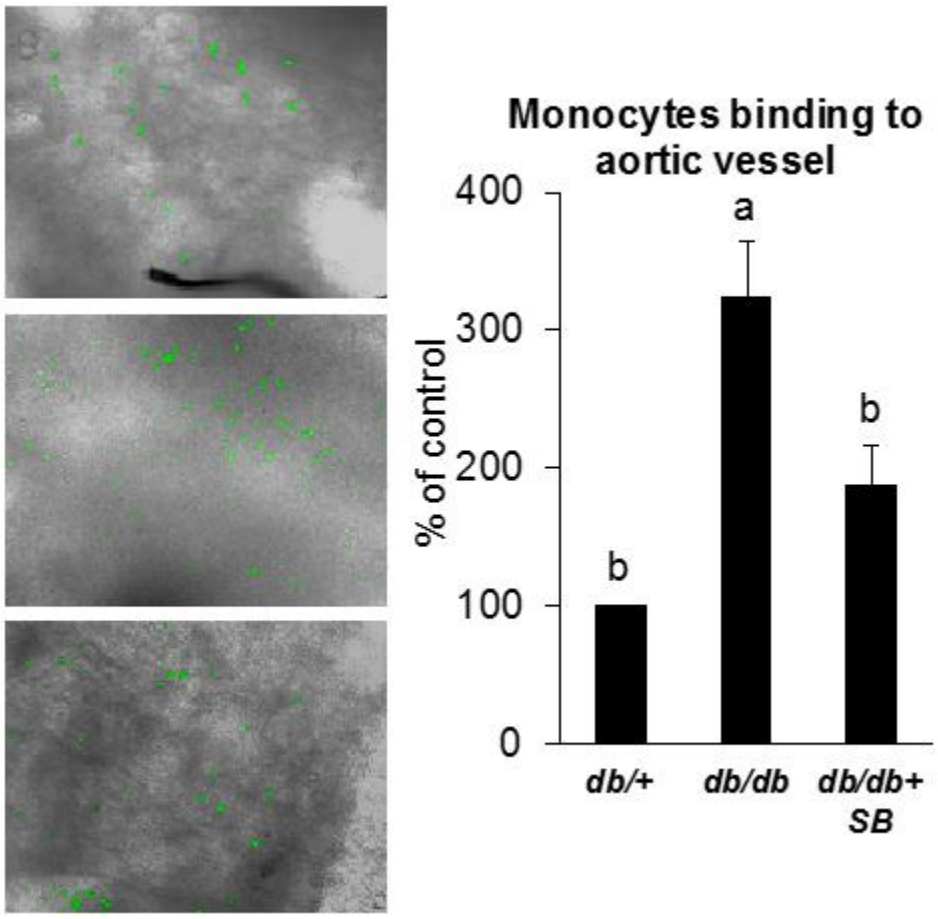

Figure 7. Monocyte Binding to Aortic Vessel. Strawberry supplementation reduces monocyte binding to aortic vessel isolated from diabetic mice *(n=6)*. *db/+*: control mice; *db/db*: diabetic mice; *db/db*+SB: diabetic mice treated with strawberry. Values are mean ± SEM. Means without a common letter differ, $P < 0.05$.

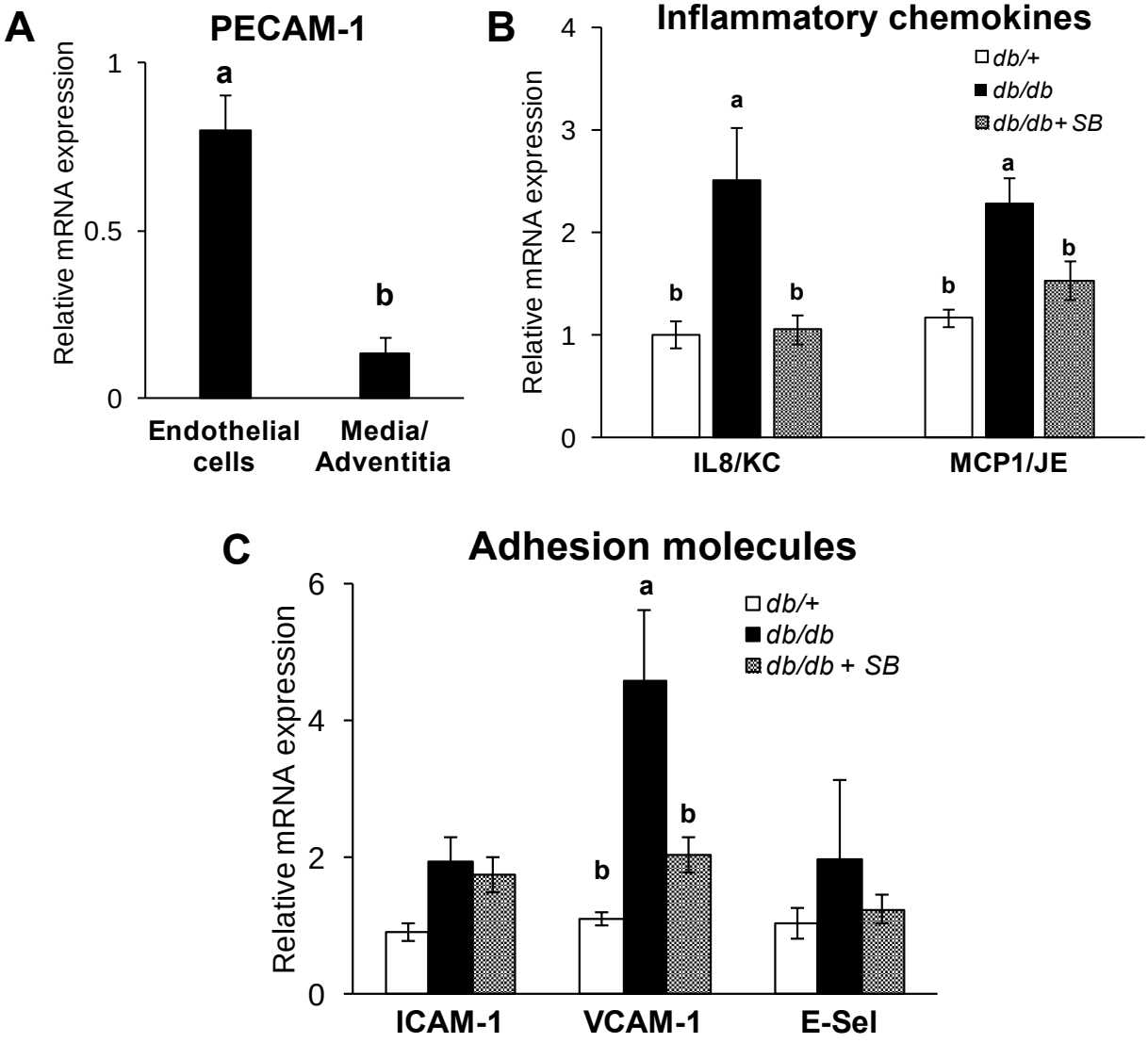

Figure 8. PECAM-1, inflammatory Chemokines and Adhesion Molecules. PECAM-1 in endothelial cells (intimal fraction) indicates the purity of endothelial cells in diabetic mice *(n=4)* (A) without changing ICAM-1 or E-selectin *(n=4) (B, C)*. *db/+*: control mice; *db/db*: diabetic mice; *db/db*+SB: diabetic mice treated with strawberry. Values are mean ± SEM. Means without a common letter differ, $P < 0.05$.

CHAPTER 4

DISCUSSION

4.1. Summary of key findings

Diabetes in its connection to cardiovascular complications and deaths is a major health concern. Uncovering strategies to mitigate the devastating effects of diabetes mellitus on vascular health is a primary focus for this project. Strawberry consumption is a promising point of focus as it has shown positive correlation with cardiovascular health outcomes in epidemiological, clinical, and animal studies (Cassidy, et al., 2011; Cassidy, et al., 2013; Giampieri, et al., 2015). Our research tested the hypothesis that dietary strawberry at a nutritional dose ameliorates vascular complications in diabetic *db/db* mice. The purpose of this study was to determine whether (i) dietary supplementation of strawberry reduces blood pressure and ameliorates endothelial dysfunction in diabetic mice, (ii) dietary strawberry improves vascular inflammation in diabetic mice, and (iii) the effect of strawberry is endothelial specific. Our findings indicate that dietary strawberry reduces blood pressure, ameliorates endothelial dysfunction, improves vascular inflammation, and the vascular effect of strawberry is endothelial specific.

4.2. Dietary supplementation of strawberry reduces blood pressure and ameliorates endothelial dysfunction in diabetic mice without affecting metabolic parameters or lipid peroxidation

Endothelial dysfunction has been shown to be linked to blood pressure and atherosclerosis. Blood pressure was significantly higher in the diabetic mice compared to the control mice as reported in previous studies (Babu, Si, Fu, et al., 2012; Babu, Si, & Liu, 2012). However, dietary supplementation of strawberry reduced blood pressure in diabetic mice. In order to show that the effect might have been due to improved endothelial function, mesenteric (resistance) vessels were tested for their differential reaction from endothelium-induced relaxation. The endothelium-dependent vasorelaxation was severely impaired in diabetic mice but strawberry supplementation improved endothelial-dependent vasorelaxation in diabetic mice without affecting endothelium-independent vasorelaxation. Strawberry supplementation did not affect the metabolic parameters such as body weight, food intake, blood glucose, serum lipids, glucose or insulin tolerance in diabetic mice. This indicates that the vascular effects of strawberry are not due to secondary effects and are not mediated through improvement in these metabolic parameters. A human study showed that strawberry supplementation improves small lipoprotein particles in subjects with metabolic syndrome (Basu, et al., 2010). We did not find a difference in serum lipids. Our findings were limited as we studied total cholesterol and serum triglycerides, but did not investigate particle size. We did find a modest reduction in lipid peroxidation in strawberry-treated diabetic mice, but this was not significant. This indicates that the vascular effects of strawberry may not be mediated through suppression of lipid peroxidation.

4.3. Dietary supplementation of strawberry improves vascular inflammation in diabetic mice and the vascular effect of strawberry is endothelial specific

Inflammation is a step toward endothelial dysfunction. Monocyte binding to vessel and transformation to macrophages and foam cells is the major step toward atherosclerosis. Inflammatory chemokines and adhesion molecules such as IL8, MCP1, ICAM1, VCAM1, and E-selectin are involved in monocyte rolling, and enhanced monocyte interaction with endothelium (Cutler, et al., 2017). In the present study, mouse monocyte WEHI 78/24 cells had significantly higher binding to the aortic vessel isolated from diabetic mice as compared to the aortas of control mice. However, monocytes binding to vessel was reduced in strawberry-treated diabetic mice. We further determined that the vascular effect of strawberry is endothelial specific and is mediated through suppression of endothelial inflammatory and adhesion molecules. The mRNA expressions of MCP1/JE, IL8/KC, ICAM1, and VCAM1 were significantly increased in endothelial cells isolated from diabetic mice as compared to control mice. Strawberry supplementation significantly reduced MCP1/JE, IL8/KC and VCAM1 in diabetic mice. This is consistent with a previous study that showed strawberry supplementation reduces circulating ICAM1 in subjects with metabolic syndrome (Basu, et al., 2010). Another study (Kuntz, et al., 2016) compared gut microbe strains on anthocyanins and found a difference in adhesion molecule expression when cells were exposed to metabolized anthocyanins from a strain of gut microbes. This is an area of investigation that should be clarified with future research.

4.4. Conclusion

In conclusion, our findings indicate that dietary supplementation of strawberry reduces blood pressure, ameliorates endothelial dysfunction, improves vascular inflammation, and reduces endothelial inflammatory and adhesion molecules in diabetic mice. Importantly, the vascular beneficial effects of strawberry were achieved at a nutritional dose that is equivalent to 2 human servings of strawberry. The reduction in blood pressure could be from improved vascular function which could have been a result of decreased inflammation. Molecular mechanisms involved in the vascular effects of strawberry should be further elucidated in future studies. Strawberries may be a dietary approach to improve vascular complications in diabetes.

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